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Individual and combined toxicity of atrazine, butachlor, halosulfuron-methyl and mesotrione on the microalga *Selenastrum capricornutum*

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ABSTRACT

The importance of controlling weeds and the needs of agricultural production mean that different herbicides are used in certain agricultural applications, and so organisms in aquatic environments are often exposed to mixtures of herbicides. This study examined the single and combined toxicity of atrazine (ATR), butachlor (BUT), halosulfuron-methyl (HAL) and mesotrione (MES), widely used herbicides with different sites of action, to the microalga *Selenastrum capricornutum* after 48 h and 72 h algal growth inhibition, using the combination index (CI)-isobologram equation. The order of toxicity of single herbicides after 48 h and 72 h of exposure ranked HAL > ATR > BUT > MES. After 48 h exposure the two mixtures of BUT + MES and BUT + HAL + MES showed a synergism effect at the effect (*fa*) level of 0.5. After 72 h exposure the five mixtures of ATR + BUT, BUT + HAL, BUT + MES, HAL + MES and BUT + HAL + MES showed synergism or additive effects at *fa* 0.5. The results indicated that the increase in toxicity caused by mixtures of these herbicides could, at suitable concentrations, pose a significant hazard to microalgae in the aquatic environment. The CI-isobologram equation may be a useful tool for the assessment of herbicide toxicity in aquatic ecosystems.

1. Introduction

The water environment is often polluted by herbicides dispersed from agricultural runoff or leaching (Elliott and Cessna, 2010). They pose a risk, therefore, to organisms in the aquatic environment. Moreover, aquatic systems are not just exposed to only a single pollutant, but to a varied co-existence of different kinds of pesticides mixture (Ferreira et al., 2008; Pérez et al., 2011). Although, an herbicide dose may be low in a natural aquatic environment, in combination they can be significantly ecotoxic (Brain et al., 2004). Gregorio et al. (2012) considered that the toxicity of mixed herbicides was a critical parameter to illustrate phytoplankton changes in waters. Many herbicides are widely used poses a potential risk to aquatic communities; the mixture of herbicides may interact and prove toxic to aquatic organisms (Magnusson et al., 2010 ; Liu et al., 2013).

Microalgae are especially vulnerable to herbicides run off from agricultural impacts via (Lorente et al., 2015). The algae are the primary producers in the aquatic environment and form the base of the food chain. The balance and function of aquatic ecosystems are impacted by the diversity and biomass of microalgae, which play a significant role in the stabilization and equilibrium of the ecosystems (Qian et al., 2012).

In percent study, four herbicides which widespread use and environmental hazard were chosen for mixed toxicological prediction. Atrazine (ATR, 2-chloro-4-ethylamino-6-isopropy-lamino-1,3,5-triazine) is an s-triazine herbicide for the control of certain annual broadleaved and grass weeds (Croplife, 2006). It is a photosynthesis inhibitor which inhibits electron transport of photosystem II, leading to inhibition of photosynthesis (Knauert et al., 2008). Due to this herbicide is widely used in the world, some researchers have considered that ATR was regarded as an endocrine disrupting contaminant in the environment, which gives it the potential to risk damaging ecological systems (Dalton, 2002; Song et al., 2009). Butachlor (BUT, N-butoxymethyl-2-chloro-N-2,6-diethyl acetanilide) is a non-ionic herbicide that controls annual grasses and broadleaved weeds (Böger et al., 2000). BUT is an enzymes inhibitor that inhibits elongase, responsible for the elongation of long-chain fatty acids and geranylgernayl pyrophosphate cyclisation enzymes (Götz and Böger, 2004). As a common pollutant in water environment, BUT poses a potential risk to the non-target organisms in aquatic ecosystems (Abigail et al., 2015). Mesotrione (MES, 2-[4-methylsulfonyl-2-nitrobenzoyl] 1,3–cyclohenanedione) is a new selective herbicide used to control broadleaved weeds in corn crops (Batisson et al., 2009). MES acts to inhibit the 4-hydroxy-phenylpYruvate dioxygenase enzyme (HPPD), a component of the biochemical pathway that

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converts tyrosine to plastoquinone and α-tocopherol (Sutton et al., 2002). Although this herbicide has a low acute toxicity in rats, it is classified as hazardous to the aquatic environment (Dumas et al., 2017). Halosulfuron-methyl (HAL, {3-chloro-5- [(4, 6-dimethoxypyrimidin-2-yl) carbamoylsulfamoyl] − 1-methyl-pyrazole-4-carboxylate)}) is a sulfonylurea herbicide that inhibits the actions of weeds acetolactate synthetase (ALS) enzymes, thus stopping weeds growth (Dubelman et al., 1997). A single dose of ATR, BUT, HAL and MES and their mixtures (e.g. ATR + BUT or ATR + MES) is widely used on sugarcane and maize in China, and these herbicides are often detected and commonly recorded as contaminants in aquatic environments (Udikovi Koli et al., 2012; Caquet et al., 2013). In recent years, some research has drawn attention to combined toxicity on non-target organisms, such as by ATR + BUT, ATR + BUT + chlorpyrifos and ATR + BUT+ cadmium on earthworms (Chen et al., 2014; Wang et al., 2015) and ATR + diuron on algae (Ge et al., 2014). However, to knowledge the combined toxicity of these four herbicides on microalgae is unknown. Thus, it is important to understand the toxicity of herbicide mixtures when assessing the potential risk to aquatic organisms.

The goal of present research was to assess the equivalent toxicity of single, binary and ternary mixtures of these four herbicides to the microalga *Selenastrum capricornutum. S.capricornutum*, an easy cultivated microalga, is a common alga in testing hazardous chemicals in water (OECD, 2006; Cho et al., 2007; Gao and Tam, 2011). The results of present research may provide an invaluable scientific basis for the accurate predicted potential risk of herbicide mixtures to *S. capricornutum*.

2. Materials and methods

2.1. Chemicals

ATR (CAS-No.102029-43-6; 95%TC), BUT (CAS-No. 23184-66-9; 95% TC) and MES (CAS-No. 104206-82-8; 95% TC) were supplied by the Guangxi Chemical Research Institute (Nanning, Guangxi, China). HAL (CAS-No.100784-20-1; 96%TC) was supplied by Jiangsu Institute Economes Co., Ltd. (Changzhou, Jiangsu, China) (Table 1). Stock solutions of each of herbicide were dissolved in deionized water in analytical-grade acetone. Each stock solution was diluted to six test concentrations.

2.2. Test algae and culture

S. capricornutum is a single-celled alga often used for assessing the toxicity of contaminant in an aquatic environment, and is the recommended algae test species of the Organization for Economic Cooperation and Development (OECD) and the International Organization for Standardization (ISO) (OECD, 2006). The alga was purchased from the Freshwater Algae Culture Collection at the Institute of Hydrobiology, Wuhan, China, and cultured in the laboratory following OECD guidelines No. 201. The algae were illuminated with cool-white fluorescent light with daily cycles of 16 h light and 8 h dark, and cultured at 23 ± 1 °C in 250 mL triangular flask containing in 100 mL boron-free OECD medium. The culture medium and flask were sterilized at 121 °C, 100 Pa, for 20 min. After this, 10 mL *S. capricornutum* was added to 90 mL of the BG11 medium (initial cell concentration approximately 10^4 cells mL^{-1}).

2.3. Toxicity to algae

The toxicity to algae tests based on algae growth inhibition (OECD, 2006) was used to determine the toxicity of the four herbicides to *S. capricornutum*. A stock solution of each of the four herbicides was prepared by dissolving them in BG11 medium. 10 μl acetone was added in 100 mL BG11 medium as controls. Three replicate test groups of algae were dosed with each concentration, including the controls. The concentration of acetone in each controls and treatments is below 100 μl/L. The algae were incubated at 21–24 °C with an LED light source of 4440–8800 lx. The triangular flasks were shaken every day during all tests. The optical density (OD) of *S. capricornutum* was determined on SHIMADZU UV-2600 after incubated 48 h and 72 h at 680 nm.

A good linear relationship between cell number and OD values was observed: the relationship was determined as $y = 0.004x + 0.010$ ($R^2 = 0.999$), where y denoted cell concentration ($5*10^4$ cells mL^{-1}) and x denoted OD at 680 nm.

The toxicity of a treatment of the four herbicides was expressed as an inhibition ratio of *S. capricornutum* cell growth, calculated as:

$$A = \sum_{i=1}^{n} \frac{OD_{i+1} + OD_i + OD_{i-1} - 3OD_0}{3} \quad (1)$$

$$E = \frac{A_0 - A_1}{A_0} * 100\% \quad (2)$$

where A_0 is an average of the relative light units (RLUs) of the controls and A an average of the RLUs of the treatments with an identical concentration; OD_i (i = 1, 2, 3, …, n) the optical density at the initial time; and OD_0 the optical density at blank BG11 medium. E is the inhibition ratio.

2.4. Mixture designs

Six binary mixtures (ATR + BUT, ATR + MES, ATR + HAL, BUT + MES, BUT + HAL and MES + HAL) and four ternary mixtures (ATR + BUT + MES, ATR + BUT + HAL, BUT + MES + HAL and ATR + MES + HAL) were used to assess the interactions. *S. capricornutum* was exposed to each herbicide single and a fixed constant ratio (1:1), basing on single EC_{50} values, in their binary and ternary mixtures. The concentration of ATR was devised six concentrations (0.004, 0.0106, 0.0281, 0.0744, 0.197, 0.522 mg L^{-1}), BUT (0.01, 0.0265, 0.0702, 0.186, 0.493, 1.307 mg L^{-1}), HAL (0.00064, 0.0017, 0.00449, 0.0119, 0.0316, 0.0836 mg L^{-1}), and MES (0.416, 1.102, 2.921, 7.742, 20.515, 54.365 mg L^{-1}). Herbicides were conducted at six concentrations with a serial dilution factor of 2.65 (Liu et al., 2009; Chen et al., 2014). Each experiment contained three replicate controls.

2.5. Mixture toxicity and CI-isobologram equation to determine individual and combined toxicities

The effect of toxicity of single and mixed of herbicides to *S. capricornutum* was assessed using a median-effect equation (Chou and Talalay, 1984):

$$\frac{fa}{fu} = \left(\frac{D}{EC50}\right)^m \quad (3)$$

Table 1
Properties of four herbicides and their mode of action.

Herbicide	CAS#	Chemical class	Chemical formula	Formular weight (g/mol)	Site of action
Atrazine	1912-24-9	triazine	$C_8H_{14}ClN_5$	215.69	photosynthesis inhibitor
Butachlor	23184-66-9	acetanilide	$C_{17}H_{26}ClNO_2$	311.85	A-starch and proteinase inhibitor
Halosulfuron-methyl	100784-20-1	sulfonylurea	$C_{13}H_{15}ClN_6O_7S$	434.81	acetolactate synthetase
Mesotrione	104206-82-8	Triketone	$C_{14}H_{13}NO_7S$	339.32	4 HPPD inhibitor